Boll. Malacol., **43** (1-8): 96-102 (2007)

Cephalopod bacterial associations: characterization and isolation of the symbiotic complex in the Accessory Nidamental Glands

Delphine Pichon, Isabelle Domart-Coulon & Renata Boucher-Rodoni

DMPA - BOME - USM 401 / UMR 5178. Muséum National d'Histoire Naturelle.
55, rue Buffon, 75005 Paris - France,
pichon@mnhn.fr

Abstract
Accessory nidamental glands (ANGs) in the reproductive system of female Myopsidae and Sepioidae (Cephalopoda) host a dense bacterial community in their tubules. Bacteria associated to the ANGs were characterized morphologically and molecularly by complementary approaches: culture dependent (isolation of bacterial strains) and culture independent (direct tissue extraction, sequence comparison of gene for 16S rRNA and fluorescent *in situ* hybridization). Populations of alpha- and gamma-proteobacteria, Gram-positive bacteria, and *Cytophaga-Flavobacteria-Bacteroides* were detected in five families of European, Asian and Australian decabrachian cephalopods. In order to obtain bacterial strains difficult to propagate artificially from the host, we have established co-cultures of *Sepia officinalis* ANG cells with their native bacteria. Cells were maintained viable over one month and the co-cultures contained bacterial strains which could not be cultured in classic microbiological media. Cephalopod cell culture can be used as a new tool to study bacteria-host interactions and their role in the physiology of each partner.

Riassunto
Le ghiandole nidamentali accessorie (ANGs) del sistema riproduttivo delle femmine delle famiglie Myopsidae e Sepioidae (Cephalopoda) ospitano nei loro tubuli una densa comunità batterica. I batteri associati alle ANGs sono stati caratterizzati morfologicamente e molecolarmente mediante approcci complementari: coltura dipendenti (isolamento di ceppi batterici) e coltura dipendenti (estrazione diretta dal tessuto, confronto della sequenza del gene 16S rRNA e ibridizzazione *in situ* a fluorescenza). Sono state scoperte popolazioni di proteobatteri-alfa e gamma, batteri Gram-positivi, e di *Cytophaga-Flavobacteria-Bacteroides* in cinque famiglie di cefalopodi decabrachiati dell'Europa, dell'Asia e dell'Australia. Al fine di ottenere ceppi batterici difficili da propagare artificialmente dall'ospite, si sono fondate co-colture di cellule ANG di *Sepia officinalis* con i loro batteri originari. Le cellule sono state mantenute vitali per più di un mese e le loro co-colture contenevano ceppi batterici che non potrebbero essere tenuti in coltura con i classici metodi microbiologi. La coltura delle cellule di cefalopodi può essere usata come un nuovo strumento di studio tra le interazioni batteri-ospite e il loro ruolo nella fisiologia di ogni partner.

Keywords
Cephalopods. Bacterial symbionts. 16S rDNA. Bacterial isolation. Co-culture.

Introduction

Symbiotic relationships between invertebrates and bacteria are ubiquitous in marine environments. These symbioses are described as binary involving just one type of microorganism (Cary *et al.*, 1993; McFall-Ngai, 1999), as involving a complex and diverse bacterial consortium. In cephalopod accessory nidamental glands (ANGs) however, we have previously shown the existence of a complex consortium of bacteria (Barbieri *et al.*, 2001; Grigioni *et al.*, 2000; Pichon *et al.*, 2005a).
The female reproductive system of myopsids, sepioids and spirulids possesses a pair of accessory nidamental glands, colorless in juveniles and deep orange at sexual maturity, implicated in egg laying. Tubules of these glands are filled with a dense bacterial community responsible for the orange coloration of the glands at sexual maturity (Bloodgood *et al.*, 1977). Bacterial consortium present in these ANGs has been described in myopsids (Barbieri *et al.*, 2001, Kaufman *et al.*, 1998, Pichon *et al.*, 2005a) and in sepioids (Grigioni *et al.*, 2000, Pichon, 2005). Specifically alpha- and gamma-proteobacteria, Gram-positive bacteria and *Cytophaga-Flavobacteria-Bacteroides* are present in different cephalopod accessory nidamental glands.
Accessory nidamental glands may have a role in egg protection since these bacteria are found associated with loliginid egg cases (Kaufman *et al.*, 1998; Barbieri *et al.*, 2001; Pichon *et al.*, 2005a), but this hypothesis still awaits confirmation.
In this project, we used a comparative approach to characterize the bacterial community present in the ANGs of cephalopod decabrachians. Diversity of the bacterial consortium was revealed both by a culture independent method (sequencing of the bacterial 16S ribosomal RNA subunit gene (16S rDNA) and fluorescent *in situ* hybridization) and a cultivation approach (both microbiological and cell-culture based).

Material and methods

Specimen collection

Females belonging to five families of cephalopods were

collected from European seawater (English Channel and Mediterranean Sea), from Taiwan, Andaman Sea and east coast of Australia (**Table 1**). All tissue samples (ANGs, embryo, yolk and egg cases) were obtained by aseptic dissection, freshly dilacerated to analyze the cultivable bacterial fraction or preserved in 100% ethanol prior to molecular analysis.

Histological analysis

ANGs and eggs were fixed and dehydrated in 100% ethanol, embedded into paraffin, and sectioned for histology (7 µm).

Gram reactivity was tested on histological sections of all individuals and bacterial smears following Gerhard *et al.* (1994) protocol.

Fluorescent *in situ* hybridization (Amann *et al.*, 1990; Hahn *et al.*, 1992; Zarda *et al.*, 1997; Grigioni *et al.*, 2000) was performed on bacterial smears and on histological sections with probes specific to eukaryotic cells (EUK502: 5'-ACCAGACTTGCCCTCC-3') (Amann *et al.* 1990), to Bacteria (EUB338: 5'-GCTGCCTCCCGTAGGAGT-3') (Amann *et al.*, 1990), to alpha-proteobacteria (ALF1B: 5'-CGTTCGYTCTGAGCCAG-3'), and to gamma-proteobacteria (GAM42A: 5'-GCCTTCCCACATCGTTT-3') (Manz *et al.*, 1992). Stringency conditions were evaluated with a gradient of formamide (10-50%). Thirty percent formamide gave the optimal images (high specific fluorescent reaction, and low background fluorescence) and was chosen for all hybridizations. Control sections were hybridized without a probe to check for background autofluorescence.

Hybridization were performed in 8 µl hybridization buffer (0.9M NaCl, 20 mM Tris/HCl, 20% or 30% N-N-dimethylformamide, 0.01%SDS) and 2 µl of probe (25 ng.µl-1 labeled with fluorescein or Cy3) during 90min at 48°C. Washing lasted 20 min at 48°C (1.02M NaCl, 20 mM Tris/HCl pH 7.2, 10 mM EDTA pH8, 0.01% SDS). Ten microliter of a 0.0001% solution of 4',6-diamino-2'-phenylindole (DAPI) was applied; samples were subsequently incubated for 10 min, then rinsed with distilled water and air dried. Samples were mounted with Cityfluor immersion oil solution (Chemical Laboratory, The University Canterbury, England) and immediately observed with an epifluorescence microscope, equipped with a high-pressure mercury bulb using filter sets (Leica) for fluorescein (480/40, 527/30), for Cy3 (535/50, 610/75) and for DAPI (340-380/425nm).

Cephalopod family	Species	Localization	ANGs	Eggs
Sepiidae	*Sepia elegans*	Mediterranean Sea	+	
	Sepia officinalis	Mediterranean Sea and English Channel	+	+
	Sepia orbignyana	Mediterranean Sea	+	
	Sepia esculenta	Taiwan	+	
	Sepia recurvirostra	Taiwan	+	
	Sepia aculeata	Taiwan	+	
	Sepia madokai	Taiwan	+	
	Sepia mestus	Andaman Sea	+	
Sepiolidae	*Sepietta neglecta*	Mediterranean Sea	+	
	Sepietta obscura	Mediterranean Sea	+	
	Sepiola rondeleti	Mediterranean Sea	+	
	Euprymna hyllebergii	Andaman Sea	+	
Sepiadariidae	*Sepiadarium kochii*	Australia	+	+
Loliginidae	*Loligo vulgaris*	English Channel	+	+
	Loligo forbesi	English Channel	+	
	Photololigo chinensis	Taiwan	+	
	Photololigo duvaucelii	Taiwan	+	
	Photololigo edulis	Taiwan	+	
	Loliolus beka	Taiwan	+	
	Loliolus uyii	Taiwan	+	
	Sepioteuthis lessoniana	Australia and Taiwan	+	+
Idiosepiidae	*Idiosepius pygmaeus*	Australia and Andaman Sea	+	+

Tab. 1. Nature and origin of specimens. ANGs: Accessory Nidamental glands.

Tab. 1. Natura ed origine dei campioni. ANGs: Ghiandole Nidamentali accessorie.

Bacterial isolation and growth conditions

For the isolation of pure bacterial strains, ANGs from live mature females belonging to five cephalopod species (*Sepia officinalis*, *S. orbignyana*, *Sepiola rondeleti*, *Sepiadarium kochii* and *Sepioteuthis lessoniana*) were dissected under aseptic conditions, and washed twice in sterile water. Tissue homogenates (100 µl) were spread on culture media: Marine Agar (DIFCO 2216, 2% NaCl) and DSMZ 308 *Vibrio* Medium (10 g Tryptone, 10 g NaCl, 4 g MgCl2*6H2O, 1 g KCl, per liter distilled water, pH 7.5). The plates were incubated at 25°C for 48 h to 72 h in order to obtain a collection of cultivable bacteria. Each distinct colony morphotypes was re-isolated in pure culture on Marine Agar. Phenotypic traits were used on Marine Agar to identify common phenotypes (Krieg & Holt 1984, Holt *et al.* 1994). Representative for each phenotypes group were selected for 16S rRNA sequence analysis.

DNA extraction

Total DNA was extracted from ANGs using the DNeasy Tissue Kit (Qiagen), following the specific protocol for bacteria for all specimens studied. For bacterial strains, DNA was extracted from colonies picked and boiled in 50 µl of sterile water, prior to 16S rRNA amplification.

Amplification of the 16S rRNA gene

PCR was conducted with dNTP (0.2 mM) (Eurogentec), primers (10 µM each), Taq polymerase (2.5 U) and Buffer (10mM Tris-HCl, 15 mM MgCl2, 500 mM Cl) (A.T.G.C. Biotechnologie) in a GeneAmp Thermocycler (Perkin Elmer) with a denaturation step at 94°C for 5 min, followed by 32 cycles of 94°C (30 s), 55°C (30 s), and 72°C (1 min) and a final elongation step at 72°C for 7 min. Universal prokaryote primers were used: 27F-1385R pair (respectively *Escherichia coli* position 9 : 5'-GAGTTTGATCCTGGCTCA-3' and position 1385 : 5'-CGGTGTGTRCAAGGCCC-3'), which produced most the entire 16S rDNA (ca 1400bp). Each PCR product was visualized by 1.5% agarose gel electrophoresis.
Purified PCR products (QIAquick PCR Purification Kit, Qiagen Inc.) were cloned by insertion into plasmid vector PCR 2.1 TOPO TA Cloning (Invitrogen) following the instructions of the manufacturer. Minipreps of the clones were sequenced using M13(-20) and M13R primers (5'-GTAAAACGACGGCCAGT-3' and 5'-CAGGAAACAGCTATGAG-3'). All sequenced clones were analyzed for the presence of chimeras using the Chimera Check program (version 2.7) (Maidak *et al.* 1997) of the Ribosomal Database Project (RDP-II) and the Bellerophon program (Huber *et al.*, 2004). Sequences suspected of being chimeric were not included in further analyses. Similarity between sequences was calculated without gap penalties. Different sequences were arbitrarily clustered into operational taxonomic units (OTU) with similarities > 97%, which defined the genus of the bacteria (Stackebrandt and Goebel, 1994).

Co-culture

Whole GNAs were rinsed for 90 min at room temperature in 25 ml of modified Locke's solution (pH 7.5) with penicilline (500 U/ml), streptomycine (500 µg/ml) and bacitracine (250 U/ml) to limit contamination by surface bacteria. Tissue was then digested twice for 15 min at 25°C in modified Locke's solution (pH 7.5) containing trypsin (0.2%). Resulting suspensions were pooled and dissociated cells and native bacteria were washed once in calcium and magnesium free seawater (CaMgFSW): 14.5 g NaCl/0.38 g KCl/0.5 g Na_2SO_4/0.125 g $NaHCO_3$ /20 ml Tris-HCl 0.5 M pH 8.0 per 500 ml of deionized H_2O [28]. Cephalopod cells were enriched from the dissociated suspension by differential density sedimentation over a 2% sucrose cushion in CaMgFSW (10 min, 200 g centrifugation at room temperature). Pellet fractions enriched in cephalopod cells were then plated in co-cultures. They were rinsed once in CaMgFSW and transferred to plastic culture dishes and Lab-tek chambers (Sigma-Aldrich, St Quentin Fallavier, France), precoated with collagen 0.01% to promote adherence of cephalopod cells, in 1.5 ml of the following culture medium without antibiotics: supplemented DMEM (Gibco 41965, Invitrogen, Cergy-Pontoise, France, supplemented with 10.8 g NaCl/0.18 g KCl/0.6 g $CaCl_2$-$2H_2O$/5.1 g $MgCl_2$-$6H_2O$/0.5 g Na_2SO_4/ 2.38 g Hepes pH 7.8 per 500 ml) half diluted with Artificial Sea Water (14 g NaCl/0.38 g KCl/0.73 g $CaCl_2$-$2H_2O$/5.22 g $MgCl_2$-$6H_2O$/0.5 g Na_2SO_4/0.12 g $NaHCO_3$/2.38 g Hepes pH 7.8 per 500 ml of deionized H_2O). Final pH of the medium was 7.8. Co-cultures were incubated at 18°C with medium addition once every week. Cephalopod cell viability was determined on a haemocytometer with the trypan blue exclusion assay and the metabolic activity of the co-culture was checked with the colorimetric MTT reduction assay.
Supernatants from co-cultures (1.5 ml) were sampled at 7 and 28 days. After centrifugation at 15000 g for 5 min., DNA was extracted from the bacterial pellets, and gene coding for 16S rRNA was sequenced.

Results

Histological results

Gram staining and DAPI staining of histological sections of ANGs demonstrated that all ANG tubules were filled with bacteria (**Figure 1 a**). Fluorescent *in situ* hybridization detected a diverse consortium of long and thin rod and coccoid shaped alpha-proteobacteria, small rod and coccoid shaped gamma-proteobacteria and large coccoid-shaped gram positive bacteria.
Similar morphotypes were present in loliginids, sepiids, sepiolids, sepiadarids and idiosepiids, but quantitative differences were observed in the abundance of each bacterial group. Gram-positive bacteria were very abundant in sepiids, sepiolids and sepiadarids. Among Gram-negative bacteria, hybridization with specific probes revealed that gamma-proteobacteria were pre-

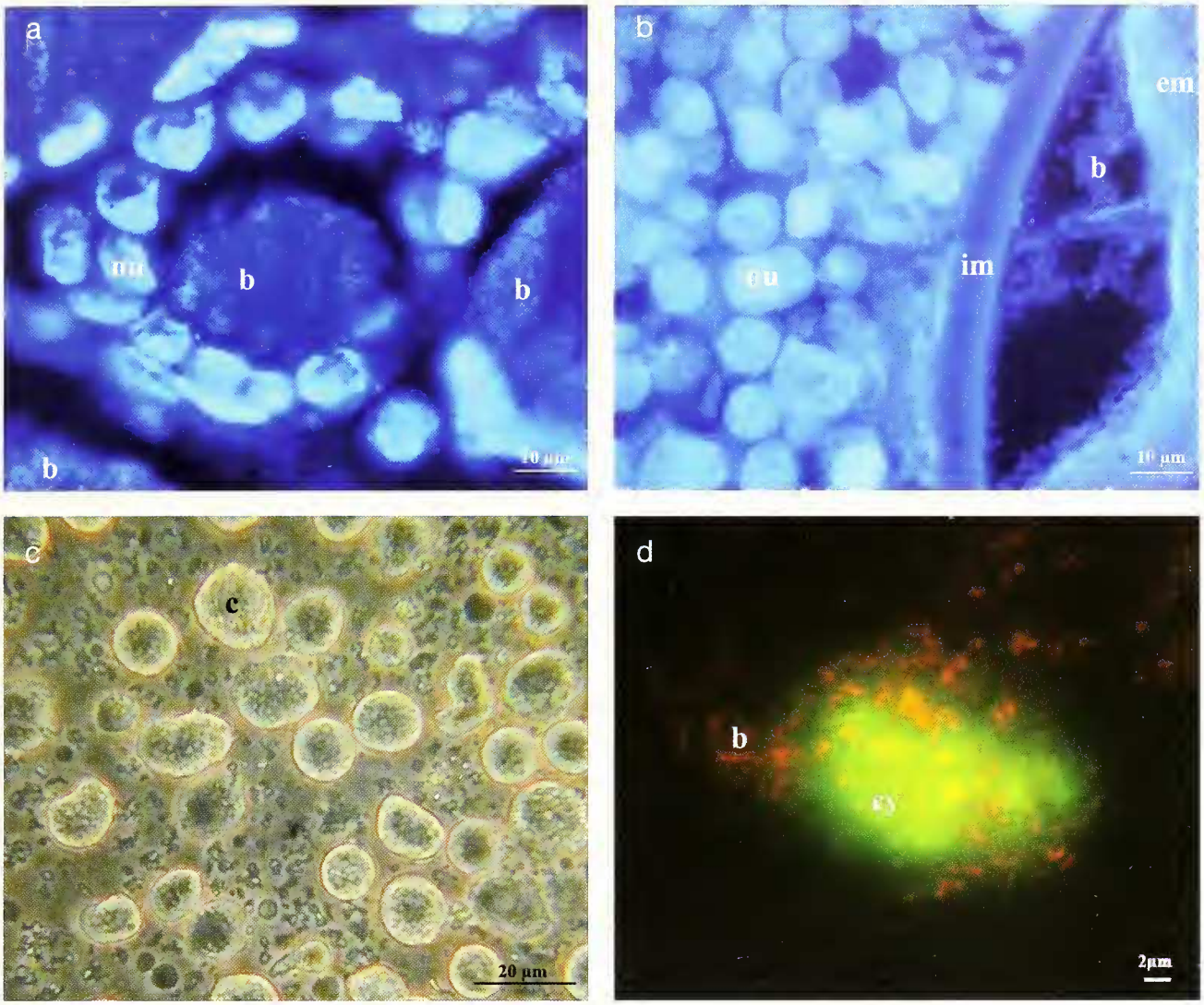

Fig. 1. a. DAPI staining of histological section of Sepia *elegans* ANGs; **b.** DAPI staining of histological section of a part of *Loligo vulgaris* egg showing embryonic tissue and membranes; **c.** ANG cells and associated bacteria co-culture; **d.** fluorescent *in situ* hybridization of cells and bacteria in co-culture after 28 days with eukaryotic probe EUK502*fluorescein and eubacteria probe EUB338*Cy3. **b.** bacteria; **c.** eukaryotic cell; cy: cytoplasm of cephalopod cell; im: internal membrane of egg cases; em: external membranes of egg cases; nu: eukaryotic nucleus.

Fig. 1. a. Colorazione DAPI di una sezione istologica di ANGs di *Sepia elegans*; **b.** Colorazione DAPI di una sezione istologica di una parte di uovo di *Loligo vulgaris* che mostra tessuto e membrane; **c.** cellule ANG e la co-coltura dei batteri associati; **d.** ibridizzazione *in situ* a fluorescenza delle cellule e dei batteri in co-coltura dopo 28 giorni con la sonda eucariotica fluorosceina EUK502* e la sonda eubatterica EUB338*Cy3. **b.** batteri; **c.** cellule eucariotiche; cy: citoplasma delle cellule di cefalopode; im: menbrana interna delle capsule ovigere; nu: nucleo eucariota.

dominant in loliginids and idiosepiids, whereas alpha-proteobacteria were predominant in the other families. No differences were observed in relation to the geography of the host.

Histological sections of eggs confirmed the presence of similar bacterial morphotypes surrounding the envelopes of the egg cases of sepioids and myopsids (**Fig. 1 b**).

Bacterial diversity

Using a culture dependant, microbiological approach, a total of 54 strains were isolated on artificial media from the ANGs of seven cephalopod species (sepioids and myopsids). Gram reactivity and physiological characteristics led to identification of 6 bacterial groups. 16rRNA gene sequencing of representative isolates for each group revealed alpha-proteobacteria (*Agrobacterium, Rhodobacter*), gamma-proteobacteria (*Vibrio, Shewanella* and *Pseudoalteromonas*) and *Cytophaga* group (**Table 2**). 80% of strains isolated on artificial media belonged to the gamma-proteobacteria group (Table 3).

Using a culture independent approach, direct extraction of the DNA from the ANGs followed by bacterial 16SrDNA sequencing confirmed the presence of the same bacterial groups as isolated in pure cultures, and allowed us to identify in the tissue three more groups: *Roseobacter* (alpha-proteobacteria), sulfur-oxidizing symbiont (gamma-proteobacteria) and Gram positive bacteria (**Table 2**). Compared to the microbiological approach, there was a shift in the relative abundance of each bacterial group revealed by the molecular approach: 79% of clones belonged to the alpha-proteobacteria group, and only 6% to the gamma-proteobacteria group (**Table 3**).

Compared to the ANGs of mature females, similar bacterial diversity was observed in the egg cases of some species as evidenced by direct 16SrRNA sequencing and by bacterial isolation on artificial media. Percentage

Group	Host species	Alpha proteobacteria			Gamma proteobacteria					
		Ag	Ro	Rb	Ps	Sh	V	Sos	Cfb	G
Sepiidae	*Sepia aculeata*			C				C	C	C
	Sepia elegans	C	C	C			C			C
	Sepia esculenta			C				C	C	C
	Sepia madokai	C		C				C		
	Sepia mestus	C		C						
	Sepia officinalis	C I	C	C I	C I	C I	C I		C I	C I
	Sepia orbignyana	C	C	C	I					
	Sepia recurvirostra			C			C			C
Sepiolidae	*Sepietta neglecta*	C		C						C
	Sepietta obscura	C	C	C	I	C				
	Sepiola rondeleti	C	C	C		I			C	
	Euprymna hyllebergii	C		C		C			C	C
Sepiadaridae	*Sepiadarium kochii*	C		C		I	C I			
Loliginidae	*Loligo forbesi*		C	C						
	Loligo pealei		C	C	I	I	I		I	
	Loligo vulgaris	C	C	C						C
	Loliolus beka	C	C	C						
	Loliolus uyii		C	C						
	Photololigo chinensis	C	C	C				C		
	Photololigo duvaucelii	C	C	C					C	
	Photololigo edulis		C	C				C	C	
	Sepioteuthis lessoniana	C		C		I	I			
Idiosepiidae	*Idiosepius pygmaeus*	C		C						C

Tab. 2. ANG bacterial diversity. C: clones; I: isolates; A: *Agrobacterium*; Ro: *Roseobacter*; Rb: *Rhodobacter*; Ps: *Pseudoalteromonas*; Sh: *Shewanella*; V: *Vibrio*; Sos: Sulfur-Oxidizing-Symbiont; Cfb: *Cytophaga-Flavobacteria-Bacteroidetes*; G: Gram Positive bacteria. The data for loliginids are from Barbieri *et al.*, 2001 and Pichon *et al.*, 2005a.

Tab. 2. ANG diversità bacterica. C: cloni; I: isolati; A: *Agrobacterium*; Ro: *Roseobacter*; Rb: *Rhodobacter*; Ps: *Pseudoalteromonas*; Sh: *Shewanella*; V: *Vibrio*; Sos: Simbionti Zulfo-Osidanti-; Cfb: *Cytophaga-Flavobacteria-Bacteroidetes*; G: batteri Gram Positivi. I dati per i loliginidi sono stati tratti da Barbieri *et al.*, 2001 e Pichon *et al.*, 2005a.

of sequence similarity between common bacterial strains from ANGs and from egg cases was in the range of 90% to 100%.

ANG cells isolated with their native bacteria and maintained *in vitro* remained viable and adherent to the collagen-coated substrate over a month. Glandular cell type was predominant in co-culture, which was polygonal, 12 µm in diameter, with voluminous nucleus (N/C ratio of 2/3) (**Fig. 1 c**). Hybridization observations (FISH with eubacterial probe) revealed proximity of bacteria with the surface of cephalopod cells (**Fig. 1 d**). Bacteria were also motile in the supernatants and surrounding the cells, and were able to form colonies when re-isolated on marine agar and incubated for 24h to 48h. Similar bacterial morphotypes in the co-cultures as in the histological sections were observed: coccoid-

Approach	Alpha-proteobacteria	Gamma-proteobacteria	Gram-positive bacteria	Cytophaga-Flavobacteria-Bacteroides
Direct sequencing	79%	6%	6%	9%
Bacterial isolation	18%	80%	—	2%
Co-Culture	43%	57%	—	—

Tab. 3. Bacterial diversity evidenced in the ANGs with the different approaches.

Tab. 3. Diversità batteriche evidenziate con l'ANGs con diversi metodi.

shaped, long and thin rod-shaped and small rod-shaped. Only gram negative bacteria were present in the co-cultures. Bacterial 16SrRNA analyses revealed presence of alpha-proteobacteria (*Roseobacter* and *Rhodobacter*) and gamma- proteobacteria (*Shewanella* and *Vibrio*) (**Table 3**).

Percentages of sequence similarity between bacterial strains detected in the ANGs and in the co-culture at 7 days and 28 days were 97 to 99.5%, and the co-cultures remained metabolically active over a month as revealed by the maintenance of the MTT reduction assay.

Discussion

Bacterial isolation and direct bacterial DNA extraction from tissue confirmed presence of 4 bacterial phyla in the ANGs of all cephalopod species studied. The closely related bacterial subgroups were present both in immature and mature ANGs, and in egg cases. Detection both in the ANGs and in the egg cases can suggest that some bacteria may be transmitted to the eggs (Barbieri *et al.*, 2001; Pichon *et al.*, 2005a). But more studies are necessary to investigate the hypothesis of vertical transmission. The potential implication of these bacteria in egg protection also needs to be further investigated.

Complementary approaches were used, which were dependant or not dependant on propagation on artificial media (microbiology) in order to alleviate bias towards specific groups. Isolation of bacterial strains detected essentially gamma-proteobacteria, whereas the direct extraction identified preferentially alpha-proteobacteria. These results confirm and explain the preliminary results obtained for myopsids and sepioids (Barbieri *et al.*, 2001; Grigioni *et al.*, 2000; Pichon *et al.*, 2005a). Indeed the microbiological approach used for *Loligo pealei* detected mostly gamma-proteobacteria, whereas the molecular approach used for both *Loligo pealei* and *Sepia officinalis* detected mostly alpha-proteobacteria.

We have developed a new approach for co-culture of ANGs cells with their associated bacteria (Pernice *et al.*, 2006). This allows the limited *ex-vivo* maintenance of a symbiotic complex which is at least partly representative of the *in situ* association. Cells and bacteria were maintained viable over one month without external contamination. In this study on the accessory nidamental glands, both *Roseobacter* and *Rhodobacter* were present in the co-culture supernatant, whereas only *Rhodobacter* had been isolated microbiologically. Some bacterial groups (including *Roseobacter*) may need the presence of the host cell to grow. We plan in the future to design experiments to investigate this hypothesis, by testing the effect of extracts from the host cells or supernatant from co-cultures on the growth of bacteria so far not amenable to microbiological cultivation. Co-cultures are indeed an interesting tool to study bacteria-cell interactions and bacteria-bacteria interactions, which are crucial for maintaining equilibrium between different populations and to control differentiation or/and proliferation of bacteria and of cells.

The bacterial assemblage our study revealed in the cephalopod accessory nidamental glands is extremely diverse, as opposed to the luminous organ symbiotic system in the sepiolid *Euprymna scolopes*, which involves just one symbiont species *Vibrio fischeri* (Nyholm and McFall-Ngai, 2004). So the difficulty of physiological studies in the ANGs is the complexity of this system. Future studies will investigate the role of each partner, especially the physiological role of the bacteria in the ANGs and in the egg cases. Several alpha-proteobacteria (*Roseobacter*, *Rhodobacter*) present in the ANGs of immature and mature females are known to produce carotenoids (Gonzales and Moran, 1997; Hiraishi *et al.*, 1995). During sexual maturation, they may be responsible for the orange coloration of the glands under physiological control by the host (Pichon *et al.*, 2005b). The role of this coloration is still unclear, it may ensure UV protection of the reproductive system, and/or promote sexual attraction of the males. The production of carotenoids appears to be an activity stimulated by hormone secretion by the optical glands (Bloodgood, 1977; Van den Branden *et al.*, 1978). The role of hormones in carotenoids production by bacterial strains needs further investigation.

Whatever the environment, whatever the developmental stage of the female, the diversity of the bacterial consortium hosted in the accessory nidamental glands is conserved, which suggest an important role for the host physiology.

Acknowledgments

The authors wish to thank: Dr. C.C. Lu and Dr M. Norman for their help during collection of specimens, and M. Pernice for his assistance. The technical help was performed by M. Martin. The authors thank the «Société Française de Malacologie» for subvention the participation to the IV International Congress of the European Malacological Societies.

References

Amann R.I., Krumholz L., Stahl D.A., 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of Bacteriology*, **172**: 762-770.

Barbieri E., Paster B.J., Hughes D., Zurek L., Moser D.P., Teske A., Sogin M.L., 2001. Phylogenetic characterization of epibiotic bacteria in the accessory nidamental gland and egg of the squid *Loligo pealei* (Cephalopoda: Loliginidae). *Environmental Microbiology*, **3**: 151-167.

Bloodgood, R., 1977. The squid accessory nidamental gland: ultrastructure and association with bacteria. *Tissue and Cell*, **9** (2): 197-208.

Cary S., Warren W., Anderson E., Giovannoni S., 1993. Identification and localization of bacterial endosymbionts in hydrothermal vent taxa with symbiont-specific polymerase chain reaction amplification and *in situ* hybridization techniques. *Molecular Marine Biology and Biotechnology*, **2** (1): 51-62.

Gerhard P., Murray R.E., Wood W.A., Krieg R. (eds), 1994. *Methods for general and molecular bacteriology*. American Society for Microbiology, Washington DC.

Gonzales J.M. and Moran A., 1997. Numerical dominance of marine bacteria in the a-subclass of the Proteobacteria in coastal seawater. *Applied and Environmental Microbiology*, **63** (11): 4237-4242.

Grigioni S., Boucher-Rodoni R., Demarta A., Tonolla M., Peduzzi R., 2000. Phylogenetic characterisation of bacterial symbionts in the accessory nidamental glands of the sepioid *S. officinalis* (Cephalopoda: Decapoda). *Marine Biology*, **136:** 217-222.

Hahn D., Amann R.I., Ludwig W., Akkermans A.D.L., Schleifer K.-H., 1992. Detection of micro-organisms in soil after in situ hybridization with rRNA-targeted fluorescently labelled nucleotides. *Journal General Microbiology*, **138:** 879-887.

Hiraishi A., Urata K. Satoh T., 1995. A new genus of marine budding phototrophic bacteria, *Rhodobium* gen. nov., which includes *Rhodobium orientis* sp. Nov. and *Rhodobium marinum* comb. nov. *International Journal of Systematic Bacteriology*, **45** (2): 226-234.

Holt J.G., Krieg P.H., Sneath A., Staley J.T., Williams S.T., 1994. *Bergey's Manual of determinative bacteriology*. The William & Wilkins, Baltimore, Maryland.

Huber T., Faulkner G., Hugenholtz P., 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics*, **20:** 2317-2319.

Kaufman M.R., Ikeda Y., Patton C., Van Dykhuizen G., Epel D., 1998. Bacterial symbionts colonize the accessory nidamental gland of the squid *Loligo opalescens* via horizontal transmission. *Biological Bulletin*, **194:** 36-43

Krieg N.R., and Holt J.G., 1884. *Bergey's Manual of systematic bacteriology*, Vol. 1. Williams & Wilkins, Baltimore, USA.

Maidak B.L., Olsen G.J., Larsen N., Overbeek R., McCaughey M.J., Woese C.R., 1997. The RDP (ribosomal Database Project). *Nucleic Acids Research*, **25:** 109-111.

Manz W., Amann R., Ludwig W., Wagner M., Schleifer K.-H., 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Systematic and Applied Microbiology*, **15:** 593-600.

Nyholm V. and McFall-Ngai M.J., 2004. The winnowing: establishing the squid-*Vibrio* symbiosis. *Nature*, **2:** 632-642.

McFall-Ngai M.J., 1999. Consequences of evolving with bacterial symbionts: insights from the squid-vibrio associations. *Annual Review of Ecology and Systematics*, **30**: 235-256.

Pichon D., 2005. *Associations symbiotiques chez les céphalopodes: étude comparative de la diversité bactérienne dans les glandes nidamentaires accessoires du système reproducteur.* Thèse de Doctorat de l'Université Paris-VI. 195p.

Pichon D., Gaia V., Norman M.D., Boucher-Rodoni R., 2005. Phylogenetic diversity of epibiotic bacteria in the accessory nidamental glands of squids (Cephalopoda: Loliginidae and Idiosepiidae). *Marine Biology*, **6:** 1323-1332.

Pichon D., Grigioni S., Favet J., Boucher-Rodoni R., 2005. Symbiotic associations between Cephalopods and the *Roseobacter* bacteria strain. *Phuket marine biological Center Research Bulletin*, **66:** 243-248.

Pernice M., Pichon D., Domart-Coulon I., Favet J., Boucher-Rodoni R., 2007. Primary co-culture as a complementary approach to explore the diversity of bacterial associations in marine invertebrates: the example of *Nautilus macromphalus* (Cephalopoda: Nautiloidea). *Marine Biology*, **150** (5): 749-757.

Stackebrandt E., Goebel B., 1994. Taxonomic note: a place for DNA-DNA reassociation and 16SrRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic bacteriology*, **44** (4): 846-849.

Van den Branden C., Richard A., Lemaire J., Decleir W., 1978. La glande nidamentaire de Sepia officinalis L.: analyses biochimiques des pigments des bactéries symbiotiques. *Annales de la Société Zoologique de Belgique*, **108**: 123-139.

Lavoro ricevuto il 6 marzo 2006
Lavoro accettato il 10 febbraio 2007